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# The effects of plant type, AMF inoculation and water regime on rhizosphere microbial communities

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## Abstract

Different plant species, water regimes and microbes in the rhizosphere might shape rhizosphere microbial communities due to their effects on root exudation patterns and interactions. In this study, we investigated whether rhizosphere microbial communities have distinct structures according to plant type (*Festuca pratensis*, *Dactylis glomerata*, or a mixture of both species), water regime (dry and wet pots) and inoculation with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (AMF). Following a 60 day pot experiment we assessed the rhizosphere microbial population structure via phospholipid fatty acids (PLFAs) and soil processes via the activity of *N*-acetyl-glucosaminidase (NAG), acid phosphatase and urease, and inorganic N and P. Higher AMF colonization was recorded in *F. pratensis*, although its root and shoot biomass was lower than in *D. glomerata*. Although growth differed between the plant types, this exerted no influence on rhizosphere microbial biomass. Low water content decreased the biomass of all microbial groups, while inoculation with AMF decreased the biomass of fungi and increased that of bacteria. For enzyme activities only urease showed a response to treatments. AMF inoculation increased available P and shifted mineral N content from nitrate to ammonium. The water regime had a dominant effect on the structure of the microbial communities, suggesting a direct effect of water on microbes. In wet soils, the structure of the microbial communities was modulated mainly by inoculation; AMF inoculated *D. glomerata* soils showed distinct communities. In dry soils, plant type exerted a profound effect on rhizosphere communities; the communities of all three plant types

differed probably due to limitations in the diffusion of nutrients or via reduced root exudation. We concluded that the relative importance of factors shaping rhizosphere microbial communities varies depending on soil moisture regime.

**Keywords:** above-below ground interactions; enzyme activities; soil nutrients; water context-specific

**Highlights**

- Microbial communities were studied in relation to water regime, plant species and AMF inoculation
- In wet soils, the microbial communities of AMF inoculated *D. glomerata* plants differed from other communities
- In dry soils, the microbial communities of *D. glomerata* and mixtures differed
- AMF increased bacterial biomass and soil P but decreased nitrate:ammonium ratio

## Introduction

Soil microbial communities are modulated by their current abiotic (temperature, humidity, pH) and biotic environment (vegetation, microbial burden, diversity of soil organisms) (Kaiser *et al.*, 2011). Changes in these conditions exert strong pressures on soil resources. The effects on soil functioning can be direct, exerted on the relative abundance and function of soil communities, or indirect through above-below ground interactions (Classen *et al.*, 2015).

Plant type can impact directly the structure of the belowground microbial communities (Ushio *et al.*, 2008). Soil microbes are carbon (C) limited (Hobbie & Hobbie, 2013); plant root exudates are the dominant source of bio-available C. Therefore, the dynamics and structure of rhizosphere microbial communities depend on the type and quality of the decaying root materials and the composition of root exudates ranging from simple sugars to complex aromatic compounds (Kos *et al.*, 2015). Moreover, the quantity of resource inputs is related to root morphology (the more the lateral roots the higher the exudation; Prikryl & Vancura (1980)), and plants could also affect microbial communities in soil via their influence on habitat properties (Eisenhauer *et al.*, 2010; Veresoglou *et al.*, 2011). For instance, a plant with a branched or a deep root system is likely to increase oxygen (O<sub>2</sub>) concentration in soil through its effect on soil penetration and aeration (Jin *et al.*, 2017).

Soil moisture level has a significant impact on the biomass and structure of soil microbial communities and their contribution to nutrient cycling (e.g. Manzoni *et al.*, 2012; Schimel 2018). Specifically, in Mediterranean soils microbial communities

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experience strong seasonal changes in water regime (Papatheodorou, 2008) including extreme desiccation during summer. Microbes have numerous strategic adaptations to deal with summer drought and these determine the structure and the stage of their community dynamics (Barnard *et al.* 2013). Zhao *et al.* (2016) attributed an increased fungal to bacterial ratio in soils receiving less precipitation to the ability of fungi to obtain water resources from micropores, more easily than bacteria. Also, distinct changes in the ratio of Gram negative bacteria:Gram positive bacteria between the wet-cold and the dry-warm season were recorded by Papatheodorou *et al.* (2012). Thus, water regime has a profound effect on the structure of the soil microbial community, because of differences in the physiology and the resource exploitation strategies between microbes. Moreover, soil moisture could have an indirect effect on microbial communities by affecting plant growth and consequently the quality and quantity of resources available to microorganisms.

Among biotic factors, the symbiosis between arbuscular mycorrhizal fungi (AMF) and roots has an important influence on microbial communities. Arbuscular mycorrhizal fungi enable plants to increase the absorption of nutrients by increasing the soil volume being exploited (Koide & Mosse, 2004), potentially having a negative effect on other soil microbes. Modifications in host plant metabolism induced by inoculation results in changes in the root exudation profile, with specific bioactive effects on organisms around the root (Sood, 2003). Furthermore, AMF inoculation alleviates water stress impacts on plants (Wu *et al.*, 2013). Accordingly, it is likely that the root colonization by AMF will interact with moisture regime and plant

species resulting in further modifications in the microbial communities. Sayer et al. (2016) provided evidence that changes in climatic variables could affect microbial communities indirectly via changes in plants' inputs, while Hawkins & Crawford (2018) proved that the microbe-plant interactions change in relation to water availability.

Apart from the effects of water regime, plant type and AMF colonization on the structural attributes of the microbial communities, a crucial question concerns the consequences for soil processes. For example, Balser & Firestone (2005) found that cyclopropyl fatty acids indicative of Gram negative bacteria were associated with nitrous oxide (N<sub>2</sub>O) production and nitrification potential while the branched fatty acids indicative of Gram positive bacteria were associated with nitrate concentration. In this study, as indices of soil processes we used the activity of *N*-acetylglucosaminidase, urease and acid phosphatase. These enzymes are involved in the transformations of C, nitrogen (N) and phosphorus (P) substrates, are mainly of microbial origin and are some of the most common indices used to assess soil functions (Ai *et al.*, 2012; Stamou *et al.*, 2017).

In the current study, we established an experiment including two grass species, *Dactylis glomerata* and *Festuca pratensis*, that coexist in the field and are similar in above-ground size and morphology (Engel & Weltzin, 2008). We suggest that these two species exhibit differences in root growth, based on the results of Mouhamadou *et al.* (2013), who found that the total root area in *D. glomerata* was about four times greater than in *F. paniculata*. We investigated the biomass and the structure of the



rhizosphere microbial communities in relation to plant type (*F. pratensis*, *D. glomerata*, mixture), water regime (dry and wet) and inoculation (*Rhizophagus irregularis*) status. In a 60-day pot experiment with an artificial soil mixture, microbial biomass and structure, soil enzymatic activities and the concentration of available P and N forms in the soil were assessed. Assuming that each plant species modulates its rhizosphere habitat properties and resources via rhizodeposition (Eisenhauer *et al.*, 2010; Van der Putten *et al.*, 2013; Veresoglou *et al.*, 2011), we hypothesized that each plant type would create a structurally unique microbial community in its rhizosphere and this community would be further mediated by soil moisture (abiotic factor) and AMF inoculation (biotic factor). Further, we tested whether this tripartite interaction influenced selected microbially mediated enzyme activities and nutrient availability.

## Materials and Methods

### *Experimental description*

The experimental design included three plant type combinations (*D. glomerata* monoculture, *F. pratensis* monoculture, and *D. glomerata* – *F. pratensis* mixture) × two AMF treatments (inoculated and non-inoculated) × two moisture regimes (wet and dry), with four replicates per treatment, giving a total of 48 pots (12.5 cm width x 20 cm height) arranged in a randomized block design. Plastic pots (2.5 l – surface sterilized with ethanol followed by 0.1% mercuric chloride) were filled with a mixture of 1:1 v/v quartz sand (Stroumboulis, Piraeus) and an amended peat product (the

medium was approximately 90% sand by mass). This mix is subsequently referred to as 'soil'. The amended peat product (AM Solvika, Lithuania) was described as peat moss amended with mineral (14% N, 16% P<sub>2</sub>O<sub>5</sub> and 18% K<sub>2</sub>O) and organic (7% N, 6% P<sub>2</sub>O<sub>5</sub> and 6% K<sub>2</sub>O) fertilisers each at a rate of 1 kg m<sup>-3</sup>. Its pH was 6.5. At the beginning of the experiment the concentration of organic C in the sand/peat mix was 0.05 g g<sup>-1</sup> dry weight whilst available N (ammonium and nitrate) and P (phosphate) concentrations were 159 and 167 µg g<sup>-1</sup> dry weight, respectively. The pH of the sand/peat mixture was 6.3.

This artificial soil was used to remove any nutrient limitation on plant growth and to avoid any potential for bias due to soil type selection. The sand/peat mixture was previously sterilized by autoclaving (4 h at 120 °C), to eradicate any indigenous AMF and other biota (Trevors, 1996). Seeds of *D. glomerata* and *F. pratensis* were surface sterilized with sodium hypochlorite 10% (v/v) for 20 min and then washed repeatedly with deionized water. The sterilized seeds were germinated in Petri dishes with water agar in the dark at 28 °C for 7 days and then transplanted into the plastic pots. Each pot contained 10 seedlings either as monocultures or five of each species in mixed cultures.

The AM fungal inoculum consisted of spores and hyphal fragments of *R. irregularis* (BEG 141, 1000 propagules/g - TERI (The Energy and Resources Institute, India)). The viability of the inoculum was tested prior to the application in 5 mixed pot cultures of *Plantago lanceolata*, *Dactylis glomerata*, and *Trifolium repens* following trap test procedures of Oehl *et al.* (2010). In all plant roots examined

the mycorrhizal colonization was >80% confirming the inoculum viability. Immediately after planting, the root system of the 7-day old *D. glomerata* and *F. pratensis* plants was inoculated with *R. irregularis* in half of the pots (20 g of the inoculum powder were added to each pot individually with water at a ratio 1:5 w/v) via a syringe to the base of plants to optimize colonization rates; the roots of the controls received the same treatment but using autoclaved inoculum powder. Care was taken to ensure no contamination of the control plants by AMF.

During the first 10 days of plant growth in the pots, the soil in each pot was watered daily at 40% water holding capacity (WHC) to enable plant establishment. Then daily watering was used to maintained pots at 80% of WHC (wet soil; gravimetric soil water content varied from 13 to 15%), or twice weekly watering maintained the rest at 25% WHC (dry soil; water content varied from 4 to 4.5%). Pots were weighed prior to each watering and the amount of added water required was estimated. Soil water content was measured twice weekly in additional pots kept under the same conditions. For the determination of soil water content, 5 g of fresh soil was dried at 105 °C for 48 hours and the water loss was estimated.

Ten days after root inoculation, a slurry was prepared using a sandy soil (65% sand, 26% silt, 9% clay; organic C 2.8%; Personal Communication) where *D. glomerata* and *F. pratensis* coexisted in a semi-natural grassland located in the Prefecture of Kilkis, 70 km north of Thessaloniki (40°56'N, 22°53'E). Soil suspension inoculants were prepared by vigorous shaking of batches consisting of 30 g of fresh soil and 200 ml of sterile, deionized water. The slurry was vacuum filtered through a

10µm Millipore, in a steri cup filter (50ml), to exclude fungal spores or hyphae. Each pot received 10 ml of this filtered suspension applied across the soil surface. The 10-day interval between inoculation and addition of microbial community is common practice (Maya & Matsubara, 2013) since soil microbiota often reduce the extent of AMF root colonization.

The experiment was conducted in a glasshouse, under natural light conditions for a 60-day period (from transplanting the seedlings to harvest; mid-June to mid-August) at the Aristotle University of Thessaloniki, Greece; day temperature ranged from 28 to 37 °C and the night temperature from 17 to 26 °C.

A destructive harvest was conducted 60 days after the planting of seedlings. In each pot, roots were separated from the soil gently by hand on a sterilized surface; rhizosphere soil was taken as that attached to roots and bulked for each individual pot; Fresh rhizosphere soil samples were sieved through a 1mm mesh to remove small roots and stored at 4°C until analysis (within a week). Samples were analyzed for microbial communities, extracellular enzyme activity and nutrient concentration. Roots were washed, dried (65 °C for 48 h), and weighed. Shoots were dried (65 °C for 48 h) and weighed. In monocultures, the total shoot and root biomass per pot was divided by the number of plants, whereas in mixtures the shoot biomass per plant was evaluated separately for *D. glomerata* and *F. pratensis*. In the case of root biomass, the total root biomass per pot was divided by the total number of plants present because distinguishing between the root systems of the two plant species was not possible.

### *Arbuscular mycorrhizal fungi (AMF) analysis*

Root samples were weighed then cleaned and stained according to Koske & Gemma (1989), modified by Orfanoudakis *et al.* (2010). The stained samples were examined under a compound microscope and AMF root colonization was evaluated according to Trouvelot *et al.* (1986). The percentage of mycorrhizal colonization and arbuscules in the root system were calculated with the MycoCalc programme (<http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html>).

### *Enzyme activity assays*

*N*-acetyl-glucosaminidase (NAG) and acid phosphatase (AP) activities were determined according to Allison & Jastrow (2006), modified for 96-well microplates. Approximately 1–2 g fresh rhizosphere soil (equivalent to 0.5 g dry weight) were added in 60 ml of 50 mM sodium acetate buffer, at pH 5, and homogenized in a blender for 1 min. Then, 50  $\mu$ L of homogenized soil slurry were combined with 150  $\mu$ L substrate solution and incubated for 3 h (NAG) or 1 h (acid phosphatase) at 21 °C under constant shaking. Substrate solutions were 2 mM *p*-nitrophenyl- $\beta$ -*N*-acetylglucosaminide for NAG and 5 mM *p*-nitrophenyl-phosphate for phosphatase, all in acetate buffer. After incubation, 100 mL of the slurry-substrate supernatant (without soil particles) were carefully transferred to another microplate for colorimetric determination of product concentrations. The *p*-nitrophenol (pNP) reaction product from the phosphatase and NAG assays was measured at 405 nm, after addition of sodium hydroxide. In each case, appropriate controls estimated the

background absorbance of the substrate and homogenate. The activity of the two enzymes was expressed as  $\mu\text{mol pNP/g d.w/ h}$ .

For the estimation of urease activity, the method of Sinsabaugh *et al.* (2000) was used. The microplate configuration was similar to that described for the NAG assay. The concentration of urea in the assay wells was 20 mM. The plates were incubated at 20 °C for approximately 18 h. Ammonium released by the reaction was quantified using colorimetric salicylate and cyanurate reagent packages from Hach. Urease activity was measured spectrophotometrically at 610 nm. Activity is expressed as  $\mu\text{mol NH}_4^+/\text{g d.w/h}$ .

#### *Chemical analyses*

$\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were determined in 2 M KCl extracts (1:10 soil dry weight solution) from rhizosphere soils by distillation and subsequent titration (Allen, 1974). For extractable P, we used the method of Olsen *et al.* (1954) as specified by Allen (1974).

#### *Phospholipid fatty acid analysis*

Extraction and analysis of phospholipids (PLFAs) was performed within one week of harvesting. Briefly this involved: (i) extraction of lipids, (ii) separation of phospholipids by column chromatography, (iii) methylation of esterified fatty acids in the phospholipid fraction, (iv) chromatographic separation and identification of the main components on a Trace GC Ultra gas chromatograph (ThermoFinnigan, San

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Jose, CA) coupled with a Trace ISQ mass spectrometry detector, a split–splitless injector, and an Xcalibur MS platform. Quantification of each fatty acid (in nmol g<sup>-1</sup>) was achieved by one-point calibration against the GC response of the internal standard 19:0 methyl ester. Under the above conditions the GC response to 19:0 methyl ester is linear in the range of 25-200 µg ml<sup>-1</sup>, with acceptable recoveries (Spyrouet *et al.*, 2009).

Overall, 22 fatty acid methyl esters were consistently present in rhizosphere samples and were considered as biomarkers for specific groups of soil microorganisms in all further analyses. According to various studies (Vestal & White, 1989; Myers *et al.*, 2001; Kourtev *et al.*, 2003; Findlay, 2004; Mastrogianni *et al.*, 2014), these biomarker PLFAs were assigned to functional groups as follows: *i*15:0, *a*15:0, 15:0, *i*16:0, 16:0, *i*17:0, 17:0 (Grampositive bacteria), 3OH12:0, 3OH14:0, 2OH16:0, 16:1 $\omega$ 9c (Gram negative bacteria); 10Me16:0, 10Me17:0, 10Me18:0 (actinobacteria). All of the above fatty acids were considered to be of bacterial origin only and their totals were chosen to represent bacterial biomass. 18:2 $\omega$ 9,12 and 18:3 $\omega$ 6c fatty acids were used as indicators of fungal biomass; 20:0, 21:0, 22:0, and 24:0 were used as indicators of microeukaryotes (algae, protozoa, nematodes; Smith *et al.*, 1986) while the PLFAs 14:0 and 18:0 are mainly of general microbial origin. The sum of all PLFAs was taken to indicate the total microbial biomass.

The ratios of PLFA biomarkers for Gram positive bacteria:Gram negative bacteria (G<sup>+</sup>/G<sup>-</sup>) and Fungi/Bacteria (F/B) ratios were calculated as broad indicators of community structure. Iso/Anteiso (Iso/Ant) and Saturated/Unsaturated (Sat/Unsat)

PLFAs were estimated as microbial stress indicators, higher values for example indicating nutrient limitation (Bach *et al.*, 2010). Iso-branched fatty acid biomass was equal to the sum of *i15:0*, *i16:0* and *i17:0*, while anteiso fatty acids were represented by *a15:0*. The degree of saturation was indicated by a number separated from the chain length number by a colon (Zelles, 1999). Thus, the biomass of saturated PLFAs was the sum of *i15:0*, *a15:0*, *15:0*, *i16:0*, *16:0*, *i17:0*, *17:0*, *14:0*, *18:0*, *20:0*, *22:0*, *23:0*, *24:0*, *10Me16:0*, *10Me17:0*, *10Me18:0*, while the total of unsaturated PLFAs was assumed to be those remaining.

#### *Statistical analyses*

Differences in the means of dependent variables across treatments were investigated by two-way analysis of variance (ANOVA). We expected that the different plant types would affect differently the soil variables causing a background variation which might override the outputs of the experimental treatments (water regime and AMF inoculation). In order to enhance the sensitivity of the ANOVA and to explore the effects of treatments as opposed to the background biological variability that the individuals of each plant type exhibit, we treated plant type as blocking variable (Webster & Lark 2018). Therefore, a Randomized Complete Block experiment with three blocks and two treatment types was set up and replicated four times. Specifically, the rhizosphere of the three plant types constituted three different blocks, while inoculation by AMF (Yes-No) and water regime (Wet-Dry) were the treatment variables. Four treatments were arranged randomly in each block, so there were



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48pots (4 treatments  $\times$  3 blocks  $\times$  4 replicates per block). The normality of the residuals, homogeneity of variances and independence of samples (ANOVA assumptions) were considered during the data analysis. In the case of deviation from ANOVA assumptions, data were transformed as appropriate. Statistical analyses were carried out with Statistica 7 package. The full ANOVA results are presented in the File S1, Supporting Information (docx file).

To seek for correlations between the variation in the individual PLFAs (Table Y; response matrix) and the tables X and W of categorical and continuous explanatory variables (predictors) respectively a RDA model was fitted to data. Plant type (*D. glomerata* and *F. pratensis* monocultures and mixtures), AMF inoculation (Yes, No) and water regime (wet, dry) were entered as discontinuous explanatory variables in the table X, the root and shoot biomasses were included in the table W of the continuous predictor variables, while Table Y stands for the structure of the microbial community in terms of individual PLFAs. Initially, the Y~X,W model was fitted to the complete data set and then the model was separately fitted to data from the wet and dry soils. The significance of the RDA model fitting was tested by a Monte Carlo test. Further, to associate the three vegetation types to specific PLFAs we based on the values of IndVal (Indicator Value, in the sense of Dufrene and Legendre (1997) which depict the membership and preference of each PLFA based on its relative occurrence and abundance. Graphically, the results were plotted on a scatter biplot where the scores of the explained data were divided by each standard

deviation (adjusted response variables). Summary statistics of RDA analysis are presented in File S2, Supporting Information (docx file)

Then, to partition the variation in the response table Y among the two tables of the explanatory data sets X and Y, we employed the variation partitioning technique as described by ter Braak & Šmilauer (2018). In brief, the variation in the table Y containing the 22 individual PLFAs (N=48) was partitioned into four fractions [a] [b] [c] and [d]. [a] represents the unique contribution of the categorical variables (plant type, AMF inoculation and water regime in the case of complete data set, plant type and AMF inoculation in the case of dry or wet soils) to the variation in the response variables (Y; individual PLFAs), [b] stands for the unique contribution of the continuous variables (root and shoot biomasses) to the variation in Y, [c] is the overlap in the explanatory power of both the categorical and continuous predictor variables, while [d] is the unexplained variation

## Results

The percentage of AMF colonization was affected by plant type ( $p < 0.05$ ), inoculation ( $p < 0.001$ ) and water regime ( $p < 0.001$ ) (Table S1.1). Higher colonization percentages were recorded in *F. pratensis* and in plants growing in dry soils whether in monoculture or mixture. The mean colonization rate in dry soils was 23.8% compared to 15.4% recorded in wet ones. In the 96% of the non-inoculated pots there were no signs of colonization while in the rest (4%), the colonization rate was negligible ( $< 0.33\%$ ).

Shoot and root biomass was affected by plant type (Tables S1.2-S1.3;  $p<0.001$  for both variables); both were higher for *D.glomerata* (Fig.1) and water. In wet soils plants had larger shoot biomass (Table S1.2;  $p<0.001$ ; Fig. 1a) while in dry soils the plants had larger root biomass (Table S1.3;  $p<0.05$ ; Fig. 1b). Increased abundance of PLFAs indicating fungi, bacteria and actinobacteria were recorded in wet soils (Tables S1.4-S1.6; Fig. 2). Moreover, fungal PLFAs were in higher abundance in non-inoculated rhizosphere soils while bacterial PLFAs were higher in inoculated ones (Fig. 2a). The F/B ratios were affected by inoculation (Table S1.8;  $p<0.001$ ), water regime ( $p<0.001$ ) and their combination ( $p<0.05$ ). Inoculation decreased F/B ratios but it was increased at higher soil moisture contents (Fig. 2c). The Sat/Unsat ratio was affected by an inoculation x water status interaction (Table S1.7;  $p=0.02$ ). Lower values of this ratio were found in non-inoculated soils, an effect particularly pronounced in wet soils (Fig. 2d).

Initially, the RDA model was applied to the complete data set. As shown by the Monte Carlo test the fitting of the entire model was highly significant ( $p<0.01$ ; Table S2.1). The amount of variation explained by both tables, X and W, [a+b+c], is 56.8% while the 43.2% remained unexplained. For the most part, the variation is accounted for by the first canonical axis (54.9%) while the second axis added a further 5.5% (Table S2.1). Water regime had the highest correlation (0.86; Table S2.2) with the first axis which accounts for most of the model variation. The variation in the table Y was portioned as indicated in Table 1. Both the variation explained by the whole model, [a+b+c], as well as the portion explained by the table X of

categorical variables (water regime, plant type and inoculation, [a]) had highly significant unique effect on the variation of the PLFAs. The negative value of [b] indicated no unique effect of the W table (root and shoot biomass). The water regime exerted a dominant effect on the structure of microbial communities and masked the effects of the other variables.

In order to study the effect of predictors other than the water regime on the microbial community, RDA analysis was applied separately on data from wet and dry soils. As shown by the Monte Carlo test (Table S2.1), the fitting of both models was significant, and the amount of variation explained by the first two canonical axes was 30.1 and 33.9 % in the wet and dry soils respectively. In particular, in wet pots the categorical and continuous variables explained the 21.4 and 10.3% of the total variation in the response variables (Table 2). As shown in Table 2, the unique contribution of the vegetation and inoculation to PLFAs variation was twofold higher (0.214) than that of the biomass variables (0.103). However, the mean squares on a per-variable ground show that the categorical and the biomass predictors were almost equally stronger predictors (0.098 and 0.078 respectively). The contribution of both the categorical ( $p < 0.05$ ) and the continuous ( $p < 0.05$ ) variables were significant.

Analysis applied to the data from wet soils revealed that AMF inoculation was correlated with the first canonical axis (0.49; Table S2.2), while *D. glomerata*, AMF inoculation and root biomass were correlated with the second axis; 0.48, 0.46 and 0.41 respectively (Table S2.2). The samples planted with *D. glomerata* were clearly separated from the remaining samples towards the upper part of the biplot (Fig. 3a).

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By contrast no separation of samples from *F. pratensis* and mixture pots was observed. Most biomarkers indicated increased colonization of the rhizosphere of *F. pratensis* in inoculated pots as indicated by the IndVal where the optima of the distribution of the PLFA biomarkers for Gram negative bacteria (3OH12:0 and 3OH14:0), Gram positive bacteria (15:0), actinobacteria (10Me18:0), and eukaryotes (20:0, 21:0, 22:0, 24:0) were recorded, while the fungal markers (18:2 $\omega$ 9,12 and 18:3 $\omega$ 6) were in larger abundance in the rhizosphere of *F. pratensis* in the non-inoculated ones (Table S2.3). Most of these biomarkers exhibited noticeable abundance for grass species mixtures, so that no distinguished ordination of the respecting samples was achieved. There were larger abundances of some biomarkers in the rhizosphere of inoculated *D. glomerata* plants, namely 2OH16:0, *i*17:0 and 10Me17:0, and almost all the corresponding samples were separately loaded towards the upper part of the second canonical axis.

The partition of variance analysis applied to the data from dry soils revealed that the unique contribution of the categorical and continuous variables to the explanation of the total variation of PLFAs was 22 and 4 % respectively, while the per-variable explanatory power of the categorical variables was sixfold higher (Table 3). Only the contribution of the categorical variables was highly significant ( $p < 0.01$ ), while the negative value of [b] indicates negligible unique effect of the W table (root and shoot biomass) to the response variables.

Plant type was correlated with both canonical axes (*D. glomerata* with the first axis, *F. pratensis* with the second one, while mixtures with both axes; Table S2.2)

while inoculation was correlated with the second axis. The separation of the samples along the first axis was clear (Fig. 3b). Samples from the mixtures were ordinated towards the left end point of the first axis, while samples for *D. glomerata* soils occupied positions towards the right end point of this axis. In relation to second axis, *F. pratensis* samples occupied the bottom part while mixture samples the upper part. According to the IndVal there were 11 biomarkers, (3OH14:0, 2OH16:0, 16:0, 14:0, 10Me17:0, 16:1 $\omega$ 9, 18:3 $\omega$ 6, 18:0, 21:0, 22:0, 24:0) that were present in larger abundance in the rhizosphere of *D. glomerata* plants, while the rhizosphere in mixtures was characterized by ten biomarkers (3OH12:0, *i*15:0, *a*15:0, 15:0, *i*16:0, *i*17:0, 10Me16:0, 10Me18:0, 18:2 $\omega$ 9,12 and 20:0; Table S2.3).

Overall, nutrient concentrations were greatly reduced compared with initial levels and these concentrations were affected by inoculation status (Tables S1.10-S1.12;  $p < 0.001$  for available P,  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ). Phosphorus and  $\text{NO}_3^-$  concentrations were larger in non-inoculated rhizosphere soils, whereas  $\text{NH}_4^+$  increased in inoculated ones (Fig. 4). Also, higher concentrations of P and  $\text{NO}_3^-$  were recorded in *D. glomerata* soils. A significant effect of soil moisture was recorded for  $\text{NH}_4^+$  (Table S1.11;  $p < 0.001$ ) which was higher in wet soils. The activity of NAG and AP was not affected by treatments (Tables S1.13-S1.14). In contrast, the activity of urease was affected by plant (Table S1.15;  $p < 0.001$ ), inoculation ( $p < 0.001$ ), water ( $p < 0.001$ ) and the inoculation x water interaction ( $p < 0.001$ ). It was greater for mixtures vs monocultures and for inoculated-wet compared to non-inoculated-dry soils (Fig. 5).

## Discussion

Water regime affected the shoot:root ratios similarly in the three plant types investigated. In wet soils, plants promote greater relative C allocation to root than to shootgrowth, resulting in higher root to shoot ratio and greater capacity to absorb water (Kozłowski & Pallardy, 2002). Silva et al. (2012) reported a reduction in shoot biomass by 28% under conditions of intermittent water limitations compared with well-irrigated conditions. The reduction in shoot biomass is related to reduced rates of C assimilation which in turn may result in lower amounts of released exudates by roots; although plant exudation responses to stress can vary, a reduction in exudation is consistent with evidence of lower microbial biomass in dry compared to wet soils. This hypothesis could explain the fact that in this study the biomass of microbial groups was affected by water regime, while plant type *per se* had no effect on rhizosphere microbial biomass. However, plant type affected root colonization rates by the AM fungus. Overall, these rates were somewhat low though not untypical of grasses in the nutrient regime followed. The colonization of *F. pratensis* roots by AMF was higher than that of *D. glomerata* although its root biomass was lower. An inverse relationship between root biomass and AMF colonization rate was recorded by Cavagnaro *et al.* (2008) for two different genotypes and was explained in terms of C allocation. According to them, the allocation of C to AMF may be approximately equal to the difference in root biomass C that was observed between the genotypes. Although the rhizosphere microbial biomass was unaffected by plant type, the biomass of roots was larger for *D. glomerata*. This is contrary to Helal & Saurbeck

(1986) who reported that plant roots induced a 197% increase in microbial biomass and Eisenhauer *et al.* (2017) who mentioned that the increase in root and shoot biomass was followed by increase in the amounts of exudates and the biomasses of fungi and bacteria. The relation between root biomass and rhizosphere microbial biomass was not analogous in this study implying that other factors than root biomass control microbial biomass; for instance the relation among root biomass and the exudate composition reported by Hertz *et al.* (2018).

Dry conditions exerted a pronounced negative effect on both bacterial and fungal biomass (Fig. 1a), despite their different sensitivity to moisture stress due to differences in their physiology (Waring *et al.*, 2013). However, a reduction of F/B ratio was recorded in dry soils. It seems that the reduction of fungal PLFAs was more pronounced compared to bacterial ones. Soil fungi that inhabit the outer surface of soil aggregates are more susceptible to drying while bacteria occupying positions into the aggregates are more tolerant to dryness (Gordon *et al.*, 2008). This is one of the mechanisms influencing the susceptibility of fungi and bacteria to soil dryness. Moreover, low moisture would limit nutrient diffusion, limiting access mainly to bacteria that have no ability to move, while low levels of soil moisture strongly inhibit extraradical hyphal density (Staddon *et al.*, 2003).

Effects of inoculation were found mainly on ratios of microbial groups. The small F/B ratio in inoculated soils resulted from a decrease in fungi and an increase of bacteria biomass, reflecting a shift in the community structure. The unexpected decrease of fungi with inoculation may be attributed to a number of factors. For



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example, pathogenic fungi such as *Fusarium* and *Rhizoctonia* (Pozo *et al.*, 2010) are suppressed by AMF due to their competition for binding sites on the root system. The literature on bacterial biomass responses to AMF is inconclusive. In the context of this particular experiment, possible explanations for the increase in biomass may be that assemblages were introduced on the walls of inoculum fungal spores acting as early inocula (Toliander *et al.*, 2006) or that there was reduced competition for bacteria because of partial fungi suppression (Pennanen *et al.*, 1999). Unlike F/B, the Sat/Unsat ratio increased in inoculated soils indicating C or nutrient limitation (Fierer *et al.*, 2003). Although nutrient limitation might be a possible explanation because fungal hyphae have large N requirements (Hodge & Fitter, 2010), the concentration of  $\text{NO}_3^-$  and P in inoculated soils remained high, suggesting that C-limitation was a more likely explanation. The establishment of the symbiotic relation between AM fungus and plant requires an expenditure of C from the plant (Délano- Frier & Tejeda-Sartorius, 2008) potentially limiting C availability to microbes in the rhizosphere. Although Atul-Nayyar *et al.* (2009) reported increased N mineralization in the presence of AMF hyphae, in this study total mineral N remained unaffected by AMF due to a trade-off between increased  $\text{NH}_4$  and decreased  $\text{NO}_3$  (see Fig. 4). This effect was more pronounced in wet compared with dry soils, suggesting that wet soils may have been oxygen limited. It is likely that at 80% WHC, ammonification proceeded while nitrification was partially inhibited (Li *et al.*, 2014).

The RDA model applied to complete data set revealed an overriding dominant effect of water regime on the structure of rhizosphere microbial communities.

Changes in the rhizosphere bacterial communities of *Cereus jamacaru* between the dry and the wet periods (Kavamura *et al.* 2013) in Brazil were attributed to different abilities of microorganisms to resist and adapt to environmental changes. Due to the dominant effect of water regime, analyses were applied separately to dry and wet soils.

In wet soils, AMF inoculation was related highly to the arrangement of samples and PLFAs in relation to the first axis while the arrangement in relation to the second axis was due to plant type, AMF inoculation and root biomass. The effect of inoculation on the structure of microbial communities in wet soils could be related to increased exudates by the extraradical mycelium (their growth is inhibited under low soil moisture) or increased root exudation, further enhanced by the colonization of roots with AMF. The rhizosphere community of inoculated *F. pratensis* soils was dominated by bacteria and microeukaryotes, the latter grazers of bacteria (Asiloglu & Murase, 2016). The paucity of fungal markers in inoculated *F. pratensis* rhizospheres supports the idea of competition between AM and free-living fungi. It is known that AMF have specific effects on the members of the microbial community (Stamou *et al.*, 2017) due to nutritional benefits and/or their involvement in competitive interactions with other microbes (Trabelsi & Mhamdi, 2013). In dry soils the structure of the communities was modulated by plant type that was highly related to both two first axes of the RDA, and inoculation which was related to the second axis. The rhizosphere communities in *D. glomerata* soils and mixtures as well as in inoculated *F. pratensis* soils and non-inoculated mixtures were clearly different. We concluded

that both factors shaped jointly the structure of the rhizosphere microbial communities while their relative importance differed in relation to water regime. Arbuscular mycorrhizal inoculation was of primary importance in wet soils while plant type was more important in dry soils. In wet soils the release of exudates from fungal extraradical structures can be transferred via water over longer distances which has a profound effect on rhizosphere communities, while in dry soils the communities are shaped mainly by root exudates due to limited transfer of solutes (Schimel & Schaeffer, 2012; Schimel, 2018). Our findings are in accordance with the results of Veresoglou *et al.* (2011) who discussed the differences between microbial communities associated with different plant species in relation to levels of nutrient input and support the idea of Hawkins & Crawford (2018) that the plant-microbe interactions are context-specific. Further reasons explaining the divergent communities in dry soils could be related to differences in plant drought tolerance (Brunner *et al.*, 2015) or the disproportionate response of the different bacterial guilds to drought (Chodak *et al.*, 2015).

Finally, despite the differentiation in rhizosphere communities in relation to the three experimental factors, the activity of NAG and AP showed no response to treatments. Different mechanisms could be proposed as possible explanations; (a) due to the increased amounts of available N and P there was no need for increased enzyme activity, (b) each microbial population produces a relatively wide range of enzymes and different microbes may produce similar enzymes (Stamou *et al.*, 2017), and (c)

the activity of extracellular enzymes stabilized in soil colloids contributes to estimated activity, but is not related to current microbial activity (Nannipieri *et al.*, 2011).

## **Conclusions**

Our findings did not support our initial hypothesis that plant species is the dominant factor affecting the structure of rhizosphere microbial communities. On the contrary, this study showed that the dominant factor was soil moisture. Soil moisture not only directly affects microbial community but also modulates the relative importance of the influence of the plant type and AMF inoculation; under sufficient water conditions AMF inoculation had the major impact while under water deficit plant type was more important. Accordingly, we suggest that discussions focusing on the factors that shape the rhizosphere microbial communities must take place within a water specific context.

## **Conflict of Interest**

The authors declare that they have no conflict of interest

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#### **Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Figures' Legends

**Figure 1.** Shoot and root biomass recorded in inoculated (+M) and non-inoculated (-M), wet (W) and dry (D) *D. glomerata* and *F. pratensis* monoculture and mixture soils (n=4, mean  $\pm$  st. error).

**Figure 2.** Biomass of fungi, bacteria (a) and actinomycetes (b) and the ratios F/B (fungi/bacteria), (c)) and Sat/Unsat (Saturated/Unsaturated; (d)) recorded in inoculated (+M) and non-inoculated (-M), wet (W) and dry (D) *D. glomerata* and *F. pratensis* monoculture and mixture soils (n=4, mean  $\pm$  st. error).

**Figure 3.** The ordination of the response variables (individual PLFAs) and samples (colored dots) from wet (a) and dry (b) soils is depicted on a RDA biplot. Dots of the same color represent samples covered by similar vegetation type. The color of PLFAs indicates preference for the vegetation type of the same color, while black dots exhibit equal preference for all three vegetation types.

**Figure 4.** Concentration of soil available P (a),  $\text{NH}_4^+$  (b) and  $\text{NO}_3^-$  (c) recorded in the soil of inoculated (+M) and non-inoculated (-M), wet (W) and dry (D), and *D. glomerata* and *F. pratensis* monoculture and mixture soils (n=4, mean  $\pm$  st. error).

**Figure 5.** Activity of urease recorded in inoculated (+M) and non-inoculated (-M), wet (W) and dry (D) *D. glomerata* and *F. pratensis* monoculture and mixture soils (n=4, mean  $\pm$  st. error).



**Table 1.** Variation partitioning results yielded by a RDA model fitted to the complete data set. Two data tables of predictors, X (Plant type, AMF inoculation, Water regime) and W (Root and Shoot biomasses) were tested for conditional effects on the table of response variables Y. [a] is the unique contribution of the Table X to the variation in the response variables Y, [b] is the unique contribution of the Table W to the variation in Y and [c] is the intersection in the explanatory power of both predictor Tables X and W.

Fraction	Variation(adj)	% of Explained	% of All	DF	Mean Square per variable
[a]	0.193	33.9	19.3	4	0.055
[b]	-0.002	-0.4	-0.2	2	0.008
[c]	0.377	66.5	37.7	--	--
Total Explained	0.568	100.0	56.8	6	0.104
All Variation	1	--	100.0	47	--
				F	P
[a+b+c]				11.3	0.002
[a]				6.0	0.002
[b]				0.9	0.486

**Table 2.** Variation partitioning results yielded by a RDA model fitted to the data set from the wet soils. Two data tables of predictors, X (Plant type and AMF inoculation) and W (Root and Shoot biomasses) were tested for conditional effects on the table of response variables Y. [a] is the unique contribution of the Table X to the variation in the response variables Y, [b] is the unique contribution of the Table W to the variation in Y and [c] is the intersection in the explanatory power of both predictor Tables X and W

Fraction	Variation(adj)	% of Explained	% of All	DF	Mean Square per variable
[a]	0.214	90.3	21.4	3	0.098
[b]	0.103	43.2	10.3	2	0.078
[c]	-0.079	-33.5	-7.9	--	--
Total Explained	0.237	100.0	23.7	5	0.081
All Variation	1	--	100.0	23	--
				F	P
[a+b+c]				2.4	0.008
[a]				3.0	0.01
[b]				2.3	0.042

**Table 3.** Variation partitioning results yielded by a RDA model fitted to the data set from the dry soils. Two data tables of predictors, X (Plant type and AMF inoculation) and W (Root and Shoot biomasses) were tested for conditional effects on the table of response variables Y. [a] is the unique contribution of the Table X to the variation in the response variables Y, [b] is the unique contribution of the Table W to the variation in Y and [c] is the intersection in the explanatory power of both predictor Tables X and W

Fraction	Variation(adj)	% of Explained	% of All	DF	Mean Square per variable
[a]	0.220	88.4	22.0	3	0.100
[b]	-0.041	-16.3	-4.1	2	0.016
[c]	0.070	27.9	7.0	--	--
Total Explained	0.249	100.0	24.9	5	0.083
All Variation	1	--	100.0	23	--
				F	P
[a+b+c]				2.5	0.002
[a]				3.1	0.004
[b]				0.5	0.920











